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The invention remains to methods for inducing mucosal immunity to an antigen and provides oligonucleotide adjuvants effective in stimulating such immunity against antigens. The adjuvants provided by the invention have little toxicity, are relatively simple to manufacture as compared to chicken lectin and other mucosal adjuvants, and possess the additional advantages of blunting the host immune response toward the T_H1 phenotype.

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METHODS AND ADJUVANTS FOR STIMULATING MUCOSAL IMMUNITY

RELATED U.S. PATENT APPLICATIONS

This is a continuation-in-part of U.S. Patent Application Serial No. 08/927,120, filed on September 5, 1997, now pending.

STATEMENT OF GOVERNMENT RIGHTS

This invention was made with Government support under Grant No. PO1 AI40682 and KO8 AI01490, awarded by the National Institutes of Health. The Government may have certain rights in this invention.

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FIELD OF THE INVENTION

The invention relates to methods and oligonucleotide compositions for use in stimulating mucosal immunity in a host.

HISTORY OF THE RELATED ART

Immunoglobulin A (IgA) antibodies and cytotoxic T cells (CTLs) are known to provide protection in mucosal tissues against a number of infectious agents, including HIV. However, effective and non-toxic means for inducing such mucosal immunity to antigens have proved elusive. For example, live attenuated vaccines produce robust immunity including mucosal IgA and CTL responses, but are difficult to produce and pose the risk of iatrogenic disease. These problems could be avoided through use of recombinant proteins from infectious agents, but immune responses to antigens delivered to mucosal surfaces are generally weak.

Mucosally active adjuvants can improve immune responses towards co-administered protein antigens substantially, but with toxicities that limit their use in humans. For example, cholera toxin is an extremely potent mucosal adjuvant, but is inherently toxic and can induce a Th₂ biased immune response which includes the development of IgE

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SUMMARY OF THE INVENTION

The invention provides a mucosally active adjuvant which not only stimulates antigen specific IgA production, but also biases the host immune response toward the Th1 phenotype, while avoiding antigen-induced IgE production. The adjuvant has little or no known toxicity in mammals, is relatively simple to manufacture and has efficacy comparable to that of cholera toxin used as a mucosal adjuvant. Indeed, strong aIgA responses can be obtained using the ISS-ODN adjuvants of the invention following a single administration.

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The mucosally active adjuvant of the invention consists of immunostimulatory oligonucleotides (ISS-ODN). Pharmaceutically acceptable compositions of ISS-ODN are provided for use in practicing the methods of the invention. The ISS-ODN of the invention include DNA or RNA oligonucleotides which are enriched with CpG dinucleotides, including those which are comprised of the primary structure 5'-Purine-(C)-(G)-Pyrimidine-Pyrimidine-3'. An especially useful form of the ISS-ODN adjuvant composition is one in which the immunizing antigen is conjugated to the ISS-ODN.

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The invention further provides means to stimulate production of secretory IgA (sIgA) in a host. To this end, ISS-ODN are administered to host mucosa before or following antigen sensitization. An especially advantageous use for this aspect of the invention is to induce immunity in a host at mucosal sites where antigens, such as allergens and pathogens, enter the body. The invention is useful in stimulating immunity in mucosae at and distal to the point of ISS-ODN administration.

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Advantageously, the mucosal immunity produced by the invention is accompanied by a shift in the host systemic immune response to the antigen away from a Th2 immune phenotype and into a Th1 immune phenotype. Thus, use of the method to boost the mucosal immune responsiveness of a host to subsequent challenge by an antigen suppresses IgE production, thereby avoiding the risk of immunization-induced anaphylaxis, in response to the antigen challenge.

Suppression of the Th2 immune phenotype is also achieved through use of the mucosal adjuvant of the invention. Thus, use of the adjuvant reduces the antigen-stimulated IL-4 and IL-5 production which accompanies canonical immunotherapy, including immunization with known adjuvants.

The ISS-ODN can also be provided in the form of a kit comprising ISS-ODN and any additional medicaments, as well as a device for delivery of the ISS-ODN to a host tissue and reagents for determining the biological effect of the ISS-ODN on the treated host.

BRIEF DESCRIPTION OF DRAWINGS

FIGURES 1a and 1b graph data confirming that mice immunized intranasally with ISS-ODN and antigen produce secretory IgA at levels comparable to those produced in response to cholera toxin (CT) and antigen immunization. This response is compared to that of mice immunized intranasally with antigen alone or with an ISS-ODN whose immunostimulatory activity has been abolished through mutation of the CpG core, neither of which groups experienced mucosal IgA production. Figure 1a reflects IgA determination in fecal samples post-immunization, in BALP samples post-immunization, and in vaginal swab samples post-immunization. Figure 1b reflect serum IgA levels in the same mice.

FIGURES 2a and 2b graph data confirming that mice immunized intranasally with ISS-ODN and antigen produce Th1 related cytokine (IPN γ) but little Th2 related cytokine (IL-4). This response is compared to the responses in mice immunized with antigen alone, an inactive ISS-ODN/antigen or CT/antigen (the latter of which induced a strong Th2

type response). Figure 2a reflects IPN γ production while Figure 2b reflects IL-4 production.

FIGURES 3a and 3b graph data confirming that mice immunized intranasally with ISS-ODN and antigen produce high titers of IgG2a (Th1) antibodies (3a), but low titers of IgG1 (Th2) antibodies (3b). This response is compared to the Th2 associated IgG1 antibodies produced in mice immunized with antigen alone, an inactive ISS-ODN/antigen or CT/antigen.

FIGURE 4 graphs data confirming that mice immunized intranasally with ISS-ODN and antigen produce cytotoxic T cells (CTLs) at levels higher than those produced in response to cholera toxin (CT) and antigen immunization. This response is compared to the low levels of CTLs produced in mice immunized with antigen alone or an inactive ISS-ODN with antigen.

FIGURE 5 graphs data confirming that mice immunized intranasally with ISS-ODN and antigen do not produce detectable titers of IgE antibodies, while mice immunized intranasally with antigen and CT produce high titers of IgE antibodies.

FIGURE 6 is a graph of data which confirm suppression of IL-4 secretion by ISS-ODN as compared to a control.

FIGURE 7 is a graph of data which confirm suppression of IL-5 secretion as a consequence of ISS-ODN mucosal adjuvant use, as compared to a control.

FIGURE 8 is a graph of data which confirm suppression of IL-10 secretion as a consequence of ISS-ODN mucosal adjuvant use, as compared to a control.

FIGURE 9 is a graph of data which confirm stimulation of IPN γ secretion as a consequence of ISS-ODN mucosal adjuvant use, as compared to a control.

FIGURE 10 is a graph of data demonstrating an ISS-ODN mediated shift to a Th1 phenotype (as indicated by IFN γ levels) in animals treated with ISS-ODN before antigen challenge (asterisked bars) or after antigen challenge.

FIGURE 11 is a graph of data demonstrating an ISS-ODN mediated boost in immune responsiveness (as indicated by increases in CD4 $^{+}$ lymphocyte proliferation) in animals treated with ISS-ODN before antigen challenge (asterisked bars) or after antigen challenge.

DETAILED DESCRIPTION OF THE INVENTION

A. Effect of ISS-ODN Adjuvants on Mucosal Immunity

1. The Mucosal Immune System

The principal port of entry for most foreign antigens into mammals are the mucosal tissues; i.e., those of the lingual/gastric, respiratory, genital, rectal and ocular organs. Immune protection in the mucosa is mediated by mucosa-associated lymphoid tissue (MALT), epithelial cells and distinct B-cell, T-cell and accessory cell sub-populations. The primary immune response which characterizes the induction of mucosal immunity to an antigen is sIgA production by activated B cells. sIgA contribute to host immunity by providing protection at the mucosal surface, thus limiting the potential for systemic involvement by the invading antigen. Terminal differentiation of B-cells into sIgA producing cells is favored by contact with IL-6, a cytokine produced in the Th1 immune phenotype. The Th1 immune phenotype also favors the induction of cell-mediated immunity, including production of the antigen specific cytotoxic T cells (CTLs) which aid in the host immune response to intracellular infections.

Although most pathogens and other foreign antigens enter the host through the mucosa, difficulties in stimulating effective mucosal immunity have limited administration of most vaccine compositions to parenteral routes. However, parenteral administration of antigens typically does not induce local immunity; e.g., production of IgA or cell-mediated immunity within the mucosa. As such, the development of host immunity following parenteral vaccination is delayed until systemic distribution of the foreign antigen is achieved.

2. Mucosal adjuvanticity of ISS-ODN

ISS-ODN administered to the mucosa according to the invention act as adjuvants and modulate the host immune response to an antigen in ways which include: (1) stimulation of local production of protective sIgA (Example I and Figure 1); (2) stimulation of cell-mediated immunity (especially CTL production; Example IV and Figure 4); and, (3) biasing of the host immune response toward the Th1 phenotype (Examples II, III and

VII, Figures 2, 3 and 6-11), with little risk of IgE mediated anaphylaxis (Example 6, Figure 5). An understanding of these phenomena is assisted by the following non-limiting definitions:

5 a. According to its art-accepted meaning, the term "adjuvant" refers to a substance "used in combination with a specific antigen that produced more immunity than the antigen alone" (Ramón, *Ann.Nat.Pariet*, 38:1-10 (1924); recently cited with approval in O'Hagen, "Recent Advances in Vaccine Adjuvants for Systemic and Mucosal Administration", *J.Pharm.Pharmacol.*, 49:1-10 (1997)).

10 b. An "enhanced" immune response is one produced by an adjuvant; i.e., a stronger immune response to an antigen than is induced by a control, generally consisting of the antigen administered alone. Using the ISS-ODN adjuvants of the invention, an enhanced immune response, as measured by antigen-specific secretory IgA (sIgA) production, can be expected to be at least 3 and as much as about 30 times the magnitude achievable through immunization with antigen alone, especially with respect to localized sIgA production. Alternatively, enhancement of the immune response by ISS-ODN of the invention, as measured by antigen-specific secretory IgA (sIgA) production, can be expected to be equivalent to or greater than (by as much as about 2 times) the magnitude achievable through immunization with antigen and cholera toxin adjuvant, especially with respect to localized sIgA production.

20 c. "Local" and "localized" refer to a mucosal tissue into which an ISS-ODN is introduced according to the invention, and fluid produced therefrom. Thus, for example, sIgA measured in bronchoalveolar lavage fluid following intranasal introduction of ISS-ODN and antigen into a host define an aspect of local mucosal immunity induced in host respiratory tissue.

25 d. "Mucosal immunity" and "mucosal immune response" refer to the response of the mammalian immune system within a mucosal tissue to an antigen introduced into that or a distant mucosal tissue, which response is characterized by,

without limitation, the production of sIgA. Mucosal immune responses may be accompanied by systemic immune responses.

5 e. "Th1 phenotype" refers to an immune phenotype mediated by the Th1 (helper T cell) subset of CD4+ lymphocytes. Th1 cells principally secrete IL-2, IFN γ and TNF β (the latter two of which mediate macrophage activation and delayed type hypersensitivity). Factors believed to favor Th1 activation resemble those induced by viral infection and include intracellular pathogens, exposure to IFN- β , IFN- α , IFN γ , IL-12 and IL-18 and exposure to low doses of antigen. Active Th1 (IFN γ) cells enhance cellular immunity (including an increase in antigen-specific CTL production) and are therefore of particular value in responding to intracellular infections.

10 f. "Th2 phenotype" refers to an immune phenotype mediated by the Th2 (helper T cell) subset of CD4+ lymphocytes. Th2 cells principally secrete IL-4 (which stimulates production of IgE antibodies), IL-5 (which stimulates granulocyte infiltration of tissue), IL-6 and IL-10. Factors believed to favor Th2 activation include exposure to IL-4, APC activity on the part of B lymphocytes and high doses of antigen. Th2 cells enhance antibody production and are therefore of value in responding to extracellular infections (albeit at the risk of anaphylactic events associated with IL-4 stimulated induction of IgE antibody production).

20 g. "Th2/Th1 switch" and "biasing toward the Th1 phenotype" refer to the occurrence of any of the following events:

(i) a reduction in levels of IL-4 measured before and after antigen-challenge; or detection of lower (or even absent) levels of IL-4 in a treated host as compared to an antigen-primed, or primed and challenged, control;

25 (ii) an increase in levels of IL-12, IL-18 and/or IFN (α , β or γ) before and after antigen challenge; or detection of higher levels of IL-12, IL-18 and/or IFN

- (α , β or γ) in an ISS-ODN treated host as compared to an antigen-primed or, primed and challenged, control;
- (iii) IgG2a antibody production in a treated host; or

- (iv) a reduction in levels of antigen-specific IgE as measured before and after antigen challenge; or detection of lower (or even absent) levels of antigen-specific IgE in an ISS-ODN treated host as compared to an antigen-primed, or primed and challenged, control.

Representative methods for determining each of these values are described in the Examples.

- (h) "Core" nucleotide sequence refers to the motif of at least six nucleotides, including at least one unmethylated CpG motif, which is present in the ISS-ODN of the invention. The relative position of each CpG sequence in ISS-ODN with immunostimulatory activity in certain mammalian species (e.g., rodents) is 5'-CG-3' (i.e., the C is in the 5' position with respect to the G in the 3' position). Many known ISS-ODN flank the CpG motif with at least two purine nucleotides (e.g., GA or AA) and at least two pyrimidine nucleotides (3'-Purine-Purine-[C]-[G]-Pyrimidine-Pyrimidine-3').

Remarkably, the invention succeeds in stimulating *algA* production and cell-mediated immunity (characterized by production of CTLs) at a magnitude comparable to that achieved through use of cholera toxin as a mucosal adjuvant (Example 1 and Figure 1). However, the invention achieves these hallmarks of mucosal immunity with little risk of the toxicity which so limits the clinical use of cholera toxin and other adjuvants in vaccine preparations (Example VI and Figure 5; also, the administration of phosphorothioate oligodeoxynucleotides to primates and, in clinical trial settings, to humans has produced no significant toxicity, even when administered at daily doses of up to 5 fold more per kilogram than those which may be used in the invention).

In addition, mucosal immunity induced in the invention is accompanied by a systemic immune response having a Th1 phenotype (see, re the Th1 environment in the mucosa:

Examples II-III and Figures 2-3; and, re the systemic Th1 phenotype of the host immune response generally: Example VII and Figures 6-11). Although the invention is not limited to any particular mechanism of action, it is conceivable that ISS-ODN facilitate uptake of exogenous antigen by antigen presenting cells for presentation through host MHC Class I processing pathways. Whatever the mechanism of action, use of ISS-ODN to boost the host's immune responsiveness to an antigen and shift the immune response toward a Th1 phenotype allows the host to be immunized at less risk of Th2 type inflammation and IgE associated anaphylactic risks (see, e.g., Example VI and Figure 5).

B. ISS-ODN Compositions

1. ISS-ODN Structure

Structurally, ISS-ODN are non-coding oligonucleotides having six or more nucleotides that may include at least one unmethylated CpG motif. The relative position of each CpG sequence in ISS-ODN with immunostimulatory activity in certain mammalian species (e.g., rodents) is 5'-CG-3' (i.e., the C is in the 5' position with respect to the G in the 3' position). Many known ISS-ODN flank the CpG motif with at least two purine nucleotides (e.g., GA or AA) and at least two pyrimidine nucleotides (3'-Purine-Purine-[C]-[G]-Pyrimidine-Pyrimidine-3'). CpG motif-containing ISS-ODN are believed to stimulate B lymphocyte proliferation (see, e.g., Krieg, *et al.*, *Nature*, 374:546-549, 1995).

The core hexamer structure of the foregoing ISS-ODN may be flanked upstream and/or downstream by any number or composition of nucleotides or nucleosides. However, ISS-ODN are at least 6 mer in length, and preferably are between 6 and 200 mer in length, to enhance uptake of the ISS-ODN into target tissues. Those of ordinary skill in the art will be familiar with, or can readily identify, reported nucleotide sequences of known ISS-ODN. For ease of reference in this regard, the following sources are especially helpful:

Yamamoto, *et al.*, *Microbiol Immunol.*, 36:983 (1992)
Ballas, *et al.*, *J Immunol.*, 157:1840 (1996)

Klimman, *et al.*, *J Immunol.*, 158:3635 (1997)

Sato, *et al.*, *Science*, 273:352 (1996)

Each of these articles are incorporated herein by reference for the purpose of illustrating the level of knowledge in the art concerning the nucleotide composition of ISS-ODN.

5 In particular, ISS-ODN useful in the invention include those which have the following hexamer nucleotide sequences:

1. ISS-ODN having "CpG" dinucleotides; and,
2. Inosine and/or uracil substitutions for nucleotides in the foregoing hexamer sequences for use as RNA ISS-ODN.

10 For example, DNA based ISS-ODN useful in the invention include those which have the following hexamer nucleotide sequences:

15 AACGTT, AGCGTC, GACGTT, GCGGCT, AACGTC, AGCGTC, GACGTC,
GGCGTC, AACGCC, AGCGCC, GACGCC, GCGGCC, AGCGCT, GACGCT,
GGCGCT, TTCGAA, GCGGTT and AACGCC (respectively, SEQ.ID.Nos. 1-
18).

ISS-ODN may be single-stranded or double-stranded DNA, single or double-stranded RNA and/or oligonucleosides. The ISS-ODN may or may not include palindromic regions. If present, a palindrome may extend only to a CpG motif. If present, in the core hexamer sequence, or may encompass more of the hexamer sequence as well as flanking nucleotide sequences.

20 The nucleotide bases of the ISS-ODN which flank the CpG motif of the core hexamer and/or make up the flanking nucleotide sequences may be any known naturally occurring bases or synthetic non-natural bases (e.g., TCAg or, in RNA, UACGf). Oligonucleosides may be incorporated into the internal region and/or termini of the ISS-ODN using conventional techniques for use as attachment points for other compounds

(e.g., peptides). The base(s), sugar moiety, phosphate groups and termini of the ISS-ODN may also be modified in any manner known to those of ordinary skill in the art to construct an ISS-ODN having properties desired in addition to the described activity of the ISS-ODN. For example, sugar moieties may be attached to nucleotide bases of ISS-ODN in any steric configuration.

5 In addition, backbone phosphate group modifications (e.g., methylphosphonate, phosphorothioate, phosphoramidate and phosphorodithioate internucleotide linkages) can confer anti-microbial activity on the ISS-ODN and enhance their stability *in vivo*, making them particularly useful in therapeutic applications. A particularly useful phosphate group modification is the conversion to the phosphorothioate or phosphorodithioate forms of the ISS-ODN oligonucleotides. In addition to their potentially anti-microbial properties, phosphorothioates and phosphorodithioates are more resistant to degradation *in vivo* than their unmodified oligonucleotide counterparts, making the ISS-ODN of the invention more available to the host.

15 2. Synthesis of, and Screening for, ISS-ODN

ISS-ODN can be synthesized using techniques and nucleic acid synthesis equipment which are well-known in the art. For reference in this regard, see, e.g., Ausubel, *et al.*, *Current Protocols in Molecular Biology*, Chn. 2 and 4 (Wiley Interscience, 1989); Maniatis, *et al.*, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., New York, 1982); U.S. Patent No. 4,458,066 and U.S. Patent No. 4,650,675. These references are incorporated herein by reference for the sole purpose of demonstrating knowledge in the art concerning production of synthetic oligonucleotides. Because the ISS-ODN is non-coding, there is no concern about maintaining an open reading frame during synthesis.

25 Alternatively, ISS-ODN or ISS enriched DNA may be isolated from microbial species (especially mycobacteria) using techniques well-known in the art, such as nucleic acid purification or hybridization. Preferably, such isolated ISS-ODN will be purified to a substantially pure state, i.e., to be free of endogenous contaminants, such as lipopolysaccharides. ISS-ODN isolated as part of a larger polynucleotide can be reduced

to the desired length by techniques well known in the art, such as by endonuclease digestion. Those of ordinary skill in the art will be familiar with, or can readily ascertain, techniques suitable for isolation, purification and digestion of polynucleotides to obtain ISS-ODN of potential use in the invention.

5 Confirmation that a particular oligonucleotide has the properties of an ISS-ODN useful in the invention can be obtained by evaluating whether the ISS-ODN affects cytokine secretion and IgG antibody isotype production as described in Section A.2(e), above. Details of *in vitro* techniques useful in making such an evaluation are given in the Examples; those of ordinary skill in the art will also know of, or can readily ascertain, other methods for measuring cytokine secretion and antibody production along the parameters taught herein.

For use in the methods of the invention, the ISS-ODN adjuvants of the invention will take the form of free ISS-ODN oligonucleotides, ISS-ODN oligonucleotide-peptide conjugates and ISS-containing recombinant expression vectors (data regarding the activity of ISS-ODN conjugates and ISS-ODN vectors are set forth in co-pending, commonly assigned U.S. patent applications Serial Nos. 60/028,118 and 08/593,554; data from which is incorporated herein by reference to demonstrate ISS-ODN immunostimulatory activity *in vivo*). In a vaccine composition, antigen may be co-delivered (separately or in an admixture with free oligonucleotides), expressed recombinantly from a plasmid (especially one containing the ISS-ODN moiety in the backbone) or, most efficaciously, the antigen is conjugated to the ISS-ODN.

Examples of other useful conjugate partners include any immunogenic antigen (including allergens, live and attenuated viral particles and tumor antigens), targeting peptides (such as receptor ligands, antibodies and antibody fragments, hormones and enzymes), non-peptidic antigens (coupled via a peptide linkage, such as lipids, polysaccharides, glycoproteins, gangliosides and the like) and cytokines (including interleukins, interferons, erythropoietin, tumor necrosis factor and colony stimulating factors). Such conjugate partners can be prepared according to conventional techniques (e.g., peptide synthesis) and many are commercially available.

Those of ordinary skill in the art will also be familiar with, or can readily determine, methods useful in preparing oligonucleotide-peptide conjugates. Conjugation can be accomplished at either termini of the ISS-ODN or at a suitably modified base in an internal position (e.g., a cytosine or uracil). For reference, methods for conjugating oligonucleotides to proteins and to oligosaccharide moieties of Ig are known (see, e.g., O'Shannessy, *et al.*, *J. Applied Biochem.*, 7:347 (1985), the disclosure of which is incorporated herein by reference solely to illustrate the standard level of knowledge in the art concerning oligonucleotide conjugation). Another useful reference is Kessler: "Nonradioactive Labeling Methods for Nucleic Acids", in Kricka (ed.), *Nonisotopic DNA Probe Techniques* (Acad. Press, 1992)).

Briefly, examples of known, suitable conjugation methods include: conjugation through 3' attachment via solid support chemistry (see, e.g., Haralambidis, *et al.*, *Nuc. Acids Res.*, 18:493 (1990) and Haralambidis, *et al.*, *Nuc. Acids Res.*, 18:501 (1990) [solid support synthesis of peptide partner]; Zuckermann, *et al.*, *Nuc. Acids Res.*, 15:5305 (1987); Carey, *et al.*, *Science*, 238:1401 (1987) and Nelson, *et al.*, *Nuc. Acids Res.*, 17:1781 (1989) [solid support synthesis of oligonucleotide partner]. Amino-amino group linkages may be performed as described in Benoit, *et al.*, *Neuromethods*, 6:43 (1987), while thiol-carboxyl group linkages may be performed as described in Sinah, *et al.*, *Oligonucleotide Analogues: A Practical Approach* (IRL Press, 1991). In these latter methods, the oligonucleotide partner is synthesized on a solid support and a linking group comprising a protected amine, thiol or carboxyl group opposite a phosphoramidite is covalently attached to the 5'-hydroxyl (see e.g., U.S. Patent Nos. 4,849,513; 5,015,733; 5,118,800 and 5,118,802).

Linkage of the oligonucleotide partner to a peptide may also be made via incorporation of a linker arm (e.g., amine or carboxyl group) to a modified cytosine or uracil base (see, e.g., Ruth, *4th Annual Congress for Recombinant DNA Research* at 123). Affinity linkages (e.g., biotin-streptavidin) may also be used (see, e.g., Rogot, *et al.*, *Nuc. Acids Res.*, 17:7643 (1989)).

Methods for linking oligonucleotides to lipids are also known and include synthesis of oligo-phospholipid conjugates (see e.g., Yamagawa, *et al.*, *Nuc. Acids Symp. Ser.*, 19:189 (1988)), synthesis of oligo-fatty acids conjugates (see e.g., Grabarek, *et al.*, *Anal. Biochem.*, 185:131 (1990)) and oligo-sterol conjugates (see e.g., Boujrad, *et al.*, *Proc. Natl. Acad. Sci. USA*, 90:5728 (1993)).

Each of the foregoing references is incorporated herein by reference for the sole purpose of illustrating the level of knowledge and skill in the art with respect to oligonucleotide conjugation methods.

Co-administration of a peptide drug with an ISS-ODN according to the invention may also be achieved by incorporating the ISS-ODN *in cis* or *in trans* into a recombinant expression vector (plasmid, cosmid, virus or retrovirus) which codes for any therapeutically beneficial protein deliverable by a recombinant expression vector. If incorporation of an ISS-ODN into an expression vector for use in practicing the invention is desired, such incorporation may be accomplished using conventional techniques which do not require detailed explanation to one of ordinary skill in the art. For review, however, those of ordinary skill may wish to consult Ausubel, *Current Protocols in Molecular Biology*, *supra*.

Briefly, construction of recombinant expression vectors (including those which do not code for any protein and are used as carriers for ISS-ODN) employs standard ligation techniques. For analysis to confirm correct sequences in vectors constructed, the ligation mixtures may be used to transform a host cell and successful transformants selected by antibiotic resistance where appropriate. Vectors from the transformants are prepared, analyzed by restriction and/or sequenced by, for example, the method of Messing, *et al.*, (*Nucleic Acids Res.*, 9:309, 1981), the method of Maxam, *et al.*, (*Method in Enzymology*, 65:499, 1980), or other suitable methods which will be known to those skilled in the art. Size separation of cleaved fragments is performed using conventional gel electrophoresis as described, for example, by Maniatis, *et al.*, (*Molecular Cloning*, pp. 133-134, 1982).

Host cells may be transformed with expression vectors and cultured in conventional nutrient media modified as is appropriate for inducing promoters, selecting transformants or amplifying genes. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

If a recombinant expression vector is utilized as a carrier for the ISS-ODN of the invention, plasmids and cosmids are particularly preferred for their lack of pathogenicity. However, plasmids and cosmids are subject to degradation *in vivo* more quickly than viruses. Alternatively, viral vectors that can be utilized in the invention include adenovirus, adeno-associated virus, herpes virus, vaccinia or an RNA virus such as a retrovirus. Of the viral vector alternatives, adeno-associated viruses would possess the advantage of low pathogenicity. The relatively low capacity of adeno-associated viruses for insertion of foreign genes would pose no problem in this context due to the relatively small size in which ISS-ODN of the invention can be synthesized.

If modification of the phosphate group of an ISS-ODN is desired (e.g., to increase its bioavailability), the techniques for making phosphate group modifications to oligonucleotides are known in the art and do not require detailed explanation. For review of one such useful technique, the intermediate phosphate triester for the target oligonucleotide product is prepared and oxidized to the naturally occurring phosphate triester with aqueous iodine or with other agents, such as anhydrous sulfur. The resulting oligonucleotide phosphorimidates can be treated with sulfur to yield phosphorothioates. The same general technique (excepting the sulfur treatment step) can be applied to yield methylphosphonamides from methylphosphonates. For more details concerning phosphate group modification techniques, those of ordinary skill in the art may wish to consult U.S. Patent Nos. 4,425,732; 4,458,066; 5,218,103 and 5,453,496, as well as *Tetrahedron Lett.* at 21:4149 (1993), 7:5575 (1986), 25:1437 (1984) and *Journal Am. Chem. Soc.*, 93:6557 (1987), the disclosures of which are incorporated herein for the sole purpose of illustrating the standard level of knowledge in the art concerning preparation of these compounds.

C. Methods for Stimulating Mucosal Immunity Through Use of the ISS-ODN Adjuvants of the Invention

1. Methods and Routes for Administration of ISS-ODN to a Host

The ISS-ODN of the invention are administered to a host using any available method and route suitable for drug delivery, including *ex vivo* methods (e.g., delivery of cells incubated or transfected with an ISS-ODN). Those of ordinary skill in the clinical arts will be familiar with, or can readily ascertain, means for drug delivery into mucosa. A useful reference in this regard is Chien, *Novel Drug Delivery Systems*, Chapters 3 through 6 and 9 (Marcel Dekker, 1992), which chapters are incorporated herein. For review, however, exemplary methods and routes of drug delivery useful in the invention are briefly discussed below.

Intranasal administration means are particularly useful in addressing respiratory inflammation, particularly inflammation mediated by antigens transmitted from the nasal passages into the trachea or bronchioli. Such means include inhalation of aerosol suspensions or insufflation of the adjuvant compositions of the invention. Nebulizer devices suitable for delivery of pharmaceutical compositions to the nasal mucosa, trachea and bronchioli are well-known in the art and will therefore not be described in detail here.

For rectal and vaginal delivery, suppository formulations and polymeric delivery devices are useful (see, e.g., the devices described in Chien, *et al.*, *J. Pharm. Sci.*, 64:1776 (1975)).

Materials and structures utilized for the delivery of contraceptive drugs to vaginal tissues (e.g., contraceptive vaginal rings used for delivery of progesterone) may also be utilized in the invention.

Pharmaceutical polymers with mucoadhesive properties are also effective means of delivering drugs to the mucosae. Examples of such polymers include carboxymethylcellulose, carboxypolypyrrolidone, polyacrylamide and sodium alginate. Encapsulation of an adjuvant composition of the invention within a mucoadhesive

polymer is an especially useful method for delivery of the composition to the gastric mucosa.

Topical administration directly to mucosal tissues is convenient and may be achieved using simple adjuvant solutions or cream preparations. Absorption promoters, such as sodium glycocholate, may be used to enhance uptake of the adjuvant preparation.

Ophthalmic administration (e.g., for treatment of allergic conjunctivitis) involves invasive or topical application of a pharmaceutical preparation to the eye. Eye drops, topical creams and injectable liquids are all examples of suitable means for delivering drugs to the eye.

A colloidal dispersion system may be used for targeted delivery of the ISS-ODN to mucosae. Colloidal dispersion systems include macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes; however, in using lipid-based formulations, the lipid character of mucosae, which may be incompatible with lipid-based pharmaceutical delivery, must be taken into account.

2. Dosing Parameters for ISS-ODN Adjuvants

A particular advantage of the ISS-ODN of the invention is their capacity to exert an adjuvant effect even at relatively minute dosages. Although the dosage used will vary depending on the clinical goals to be achieved, a suitable dosage range is one which provides up to about 1-1000 μg of ISS-ODN/ml of carrier in a single dosage. In view of the teaching provided by this disclosure, those of ordinary skill in the clinical arts will be familiar with, or can readily ascertain, suitable parameters for administration of ISS-ODN according to the invention.

Clinically, it may be advisable to administer the ISS-ODN in a low dosage (e.g., about 1 μg /ml to about 50 μg /ml), then increase the dosage as needed to achieve the desired therapeutic goal. Based on current studies, ISS-ODN are believed to have little or no toxicity at these dosage levels.

3. *Pharmaceutical Compositions of ISS-ODN*

If to be delivered without use of a vector or other delivery system, ISS-ODN will be prepared in a pharmaceutically acceptable composition. Pharmaceutically acceptable carriers preferred for use with the ISS-ODN of the invention may include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcohol/water aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, antioxidants, chelating agents, and inert gases and the like. A composition of ISS-ODN may also be lyophilized using means well known in the art, for subsequent reconstitution and use according to the invention.

Absorption promoters, detergents and chemical irritants (e.g., keratinolytic agents) can enhance transmission of an ISS-ODN composition into a target tissue. For reference concerning general principles regarding absorption promoters and detergents which have been used with success in mucosal delivery of organic and peptide-based drugs, see Chien, *Novel Drug Delivery Systems*, Ch. 4 (Marcel Dekker, 1992).

Examples of suitable nasal absorption promoters in particular are set forth at Chien, *supra* at Ch. 5, Tables 2 and 3; milder agents are preferred. Suitable agents for use in the method of this invention for mucosal/nasal delivery are also described in Chang, *et al.*, *Nasal Drug Delivery*, "Treatise on Controlled Drug Delivery", Ch. 9 and Table 3-4B thereof (Marcel Dekker, 1992). Suitable agents which are known to enhance absorption of drugs through skin are described in Sloan, *Use of Solubility Parameters from Regular Solution Theory to Describe Partitioning-Driven Processes*, Ch. 5, "Prodrugs: Topical and Ocular Drug Delivery" (Marcel Dekker, 1992), and at places elsewhere in the text. All of these references are incorporated herein for the sole purpose of illustrating the level of knowledge and skill in the art concerning drug delivery techniques.

D. *Kits for Use in Practicing the Methods of the Invention*

For use in the methods described above, kits are also provided by the invention. Such kits may include any or all of the following: ISS-ODN (conjugated or unconjugated); a pharmaceutically acceptable carrier (may be pre-mixed with the ISS-ODN) or suspension base for reconstituting lyophilized ISS-ODN; additional medicaments, which may include a conventional vaccine preparation for co-administration with an ISS-ODN adjuvant; a sterile vial for each ISS-ODN and additional medicament, or a single vial for mixtures thereof; device(s) for use in delivering ISS-ODN to a host; or assay reagents for detecting indicia that the immunostimulatory effects sought have been achieved in treated animals and a suitable assay device.

Examples illustrating the practice of the invention are set forth below. Data evidencing systemic responses to ISS-ODN are included for the purpose of confirming the general effect of ISS-ODN on the mammalian immune system and, to that extent, apply to the mucosal environment. The examples are for purposes of reference only and should not be construed to limit the invention, which is defined by the appended claims. All abbreviations and terms used in the examples have their expected and ordinary meaning unless otherwise specified.

EXAMPLE I
THE ISS-ODN OF THE INVENTION
ARE EFFECTIVE ADJUVANTS FOR STIMULATION
OF ANTIGEN-SPECIFIC IgA PRODUCTION

5 To demonstrate the efficacy of ISS-ODN in stimulation of IgA production to antigen, the mucosal IgA responses of mice immunized intranasally with β -galactosidase (β -gal) in the presence of either ISS-ODN or cholera toxin (CT; the most potent known mucosal adjuvant) were compared. Mice were separately immunized intranasally with antigen alone or with an ISS-ODN modified to eliminate its immunostimulatory ability (M-ISS-ODN; CpG dinucleotide motif in DY1018 substituted with a immunosuppressive CpG motif). The ISS-ODN used in these studies has the following sequence: 5- TGACTGTGTAACGTTTCGAGATGA-3. The M-ODN has the sequence: 5- TGACTGTGTAACCTTAGAGATGA-3.

10 To this end, female BALB/c mice aged 6-8 weeks were purchased from Jackson Laboratory (Bar Harbor, ME) and used in all experiments. Intranasal immunizations were performed with β -gal (50 μ g) alone, mixed with or conjugated to 50 μ g of ISS-ODN or M-ODN, or with CT (10 μ g) in 30 μ l of saline. Alternatively, mice received β -gal (200 μ g) plus ISS-ODN (50 μ g) in 50 μ l of saline injected i.d. into the base of the tail, or β -gal (200 μ g) plus ISS-ODN (50 μ g) administered intragastrically (i.g.) By blunted needles in 400 μ l of 0.2M Na bicarbonate. Mice were fasted for 4 hours before i.g. immunization. Bronchoalveolar lavage fluid (BALF) was obtained by cannulation of the trachea of sacrificed mice. The lungs were flushed with 0.8ml of PBS and the return frozen at -70°C until IgA assay. Feces and vaginal swabs were collected at 2 week intervals.

25 Serum, BALF, and fecal extraction fluid were used in ELISA assays for antigen specific immunoglobulin. Results shown in Figure 1 are expressed in units/ml based on pooled high titer anti- β -gal standards. The undiluted fecal IgA and IgG standards were given arbitrary concentrations of 2,000 and 400,000 U/ml respectively. Samples were compared to the standard curve on each plate using the DeltaSOFT II v. 3.66 program

(Biometallica, Princeton, NJ). Statistical analysis of results was conducted using Statview computer software (Abacus Concepts, Grand Rapids, MI). A 2 tailed Student's t test was used to establish p values and those ≤ 0.05 were considered significant.

Referring to Figure 1(a) and 1(b), at 7 weeks post-immunization, mice immunized with antigen alone or with M-ISS-ODN had little or no detectable levels of IgA in feces, bronchoalveolar lavage fluid (BALF) or vaginal swab samples. Mice immunized with β -gal/CT had a mean IgA level in fecal material of 599 U/ml, in (BALF) of 1432 U/ml and in vaginal swabs of 16000 U/ml. Surprisingly, IgA levels achieved in the β -gal/ISS-ODN conjugate immunized mice were comparable to the levels achieved in mice immunized with antigen and CT (without statistically significant difference); i.e., 462 U/ml in feces, 2935 U/ml in BALF and 12500 U/ml in vaginal swabs (co-delivery of ISS-ODN with antigen induced slightly lower levels of IgA production than did delivery of the ISS-ODN/antigen conjugate). The adjuvanticity necessary to achieve a mucosal immune response to antigen was therefore achieved as well by ISS-ODN as by the conventional, and more toxic, cholera toxin adjuvant.

To determine whether the adjuvanticity of ISS-ODN in mucosa was a consequence of mucosal administration, another group of mice were immunized intradermally with β -gal and ISS-ODN via intradermal (i.d.) and intragastric (i.g.) routes. These routes of immunization did not lead to mucosal IgA production. To establish whether the IgA detected in fecal material and BALF of immunized mice was actively secreted by mucosal tissue or passively diffused from serum, anti- β -gal IgA levels in serum, fecal material, and BALF were also compared. Serum IgA levels in i.n. β -gal/ISS-ODN immunized mice were 2 fold lower than in fecal material and 10 fold lower than in BALF, providing strong evidence that anti- β -gal IgA was in fact secreted by mucosal tissue (Figure 1(a)).

These results demonstrate that ISS-ODN and CT have equivalent mucosal adjuvant activity with a test antigen which has no capacity to induce mucosal IgA production when delivered alone. In addition, we show that i.d. delivery of β -gal with ISS-ODN does not

lead to a mucosal IgA response. Taken together these findings show that ISS-ODN is an excellent adjuvant for the induction of mucosal immunity when co-delivered with antigen via the mucosa (here, the nose).

EXAMPLE II
THE ISS-ODN OF THE INVENTION
ARE EFFECTIVE ADJUVANTS FOR STIMULATION
OF A Th1 BIASED SYSTEMIC IMMUNE RESPONSE

The magnitude and phenotype of the systemic immune response induced by mucosal (intranasal, i.n.) administration of an antigen (β -gal) with an ISS-ODN adjuvant was studied. For comparison, the same immune response was studied in mice who received β -gal and ISS-ODN by the intradermal (i.d.) route. Mice were separately immunized intranasally with antigen alone or with an ISS-ODN modified to eliminate its immunostimulatory ability (M-ISS-ODN; CpG dinucleotide motif IDY1018 substituted with a immunosuppressive CpG motif). The ISS-ODN used in these studies has the following sequence: 5-TGACTGTGAAAGCTTAGAGATGA-3. The M-ODN has the sequence 5-TGACTGTGAAAGCTTAGAGATGA-3.

To this end, splenocytes were harvested from the β -gal/ISS-ODN immunized mice described in the preceding example 7 weeks after immunization, incubated with β -gal, and culture supernatants assayed for the production of IFN γ and IL-4, cytokines classically associated with Th₁ and Th₂ immunity. Splenocyte cytokine profiles were conducted by incubation of 5×10^5 splenocytes in 96 well plates in a final volume of 200 μ l of supplemented RPMI 1640 with β -gal added at 10 μ g/ml at 37°C/5% CO₂. Culture supernatants were harvested at 72 hours and analyzed by ELISA. A standard curve was generated using known amounts of recombinant IL-4 (Genzyme, Cambridge, MA) and IFN γ (Pharmingen, San Diego, CA). Each culture supernatant was compared to the standard curve on the plate using the DeltaSoft II v. 3.66 program. Statistical analyses were performed as described in the preceding example.

Referring to Figure 2, splenocytes from mice immunized with β -gal and ISS-ODN via the i.n. and i.d. routes respectively produced a mean of 2084 and 1720 pg/ml of the Th1 cytokine IFN γ , but none of the Th2 stimulatory cytokine IL-4. Thus, mice immunized

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with ISS-ODN adjuvant by either a mucosal (i.n.) or systemic (i.d.) route responded to the antigen in a Th1 biased manner.

In contrast, i.n. vaccination with β -gal and CT led to splenocytes that produced a mean of 542 pg/ml of IFN γ and 73 pg/ml of IL-4 ($p = 0.05$ for both IFN γ and IL-4 compared to i.n. β -gal/ISS-ODN immunization; Figure 2). Intranasal immunization with β -gal alone or with M-ODN led to poor or undetectable antigen-specific cytokine production from splenocytes. Thus, immunization with conventional or no adjuvant favored more of a Th2 type immune response, if any, response at all, to the antigen.

EXAMPLE III
MUCOSAL IMMUNIZATION ACCORDING TO THE INVENTION
PRODUCES A Th1 TYPE IgG ISOTYPE PROFILE

IFN γ is an IgG isotype switch factor for IgG2a production (associated with a Th1 immune phenotype), while IL-4 is a switch factor for IgG1 production (associated with a Th2 immune phenotype). Consistent with the cytokine profiles induced following mucosal antigen immunization with ISS-ODN adjuvant according to the invention, mice immunized intranasally with β -gal/ISS-ODN immunized mice produced Th₁ biased serum antibody responses. However, mice immunized while i.n. β -gal/CT vaccination led to a Th₂ biased IgG subclass profile.

Specifically, at 7 weeks post intranasal immunization of mice with β -gal/ISS-ODN mean serum anti- β -gal IgG2a levels were 306,144 and 362,850 U/ml (Figure 3(a)) and anti- β -gal IgG1 levels were 5,971 and 3,676 U/ml respectively (Figure 3(b)). These differences were not statistically significant. In contrast, intranasal immunization with β -gal and CT induced mean serum IgG2a and IgG1 levels of 94,518 and 36,471 U/ml ($p = 0.005$ for IgG2a and 0.004 for IgG1 compared to i.n. β -gal/ISS-ODN immunization). Again, i.n. immunization with β -gal alone or with M-ODN led to poor or undetectable IgG responses.

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Taken together these observations show that i.n. and i.d. delivery of antigen with ISS-ODN lead to Th₁ biased cytokine and antibody profiles while i.n. β -gal/CT co-administration leads to a Th₂ biased systemic immune response. Considered in conjunction with the IgA data previously presented, it is clear that mucosal immunization with antigen and ISS-ODN beneficially biases the systemic immune response toward a Th1 response *in addition* to stimulating production of IgA.

EXAMPLE IV

MUCOSAL IMMUNIZATION ACCORDING TO THE INVENTION STIMULATES CYTOTOXIC T CELL PRODUCTION

CTL responses are characteristic of Th₁ biased immunity. However, not all Th₁ biased immune responses include the development of cytotoxic T cells and immunization with protein alone does not lead to the development of CTL activity.

For CTL assays, 7×10^6 splenocytes from immunized mice were incubated with 6×10^4 mitomycin-C treated naive splenocytes in the presence of recombinant human IL-2 and class I H2^d restricted β -gal nanopetide peptide (T-P-H-P-A-R-I-G-L). After 5 days, restimulated cells were harvested and specific lysis of target cells measured.

As can be seen in Figure 4, mice immunized with β -gal and ISS-ODN by the intranasal route had vigorous splenic CTL activity (in addition to IgA production). In particular, at an E:T ratio of 5:1, intranasal co-delivery of β -gal/ISS-ODN led to 52% specific lysis of target cells. However, i.n. β -gal/CT immunization resulted in only 3% specific lysis at the same E:T ratio ($p < 0.005$ compared to immunization with β -gal/ISS-ODN). Likewise, i.n. immunization with β -gal alone or with M-ODN led to poor or undetectable CTL responses.

These results show that β -gal/ISS-ODN co-immunization through mucosa leads to robust CTL responses, while mucosal immunization in the presence of CT adjuvant does not.

EXAMPLE V

LACK OF TOXICITY ON THE PART OF ISS-ODN

The following passages are quotations from the paper Webb, *et al.*, *Lancet*, 349:1137 (1997). The Webb, *et al.* study reported the results of a clinical trial use of an oligonucleotide having a CpG motif therein in antisense cancer therapy. The toxicity study results are directly predictive of the probable toxicity (lack thereof) of the ISS-ODN adjuvants of the invention.

"Eligible patients were men or women who had non-Hodgkin lymphoma of any histological grade with immunohistochemical evidence of overexpression of the BCL-2 protein on lymph-node biopsy samples. In addition, patients had to have relapsing disease after the completion of at least two chemotherapy regimens, a life expectancy of more than 12 weeks, normal renal and liver function, a white-blood-cell count of more than $3 \times 10^9/L$, and a platelet count of more than $100 \times 10^9/L$.

A daily dose of an 18-base, fully phosphorothioated oligonucleotide (sequence 5'-TCTCCACGGTGGCCAT-3', dissolved in isotonic normal saline) was administered as a subcutaneous continuous infusion with a portable syringe driver. This oligonucleotide was complementary to the first six codons of mRNA of the BCL-2 gene. Infusion sites were changed when we observed early signs of inflammation. Treatment-related toxicity was scored by the common toxicity criteria;...we included areas of concern pinpointed in animal and in-vitro studies...Toxicity was monitored for the first 48 h of treatment while the patients were in hospital, but thereafter on an outpatient basis. One 2-week course of treatment was given. Patients were followed up for 4 weeks after the end of treatment. If there was evidence of tumour response, a second course was considered. The initial daily dose was $4 \times 6 \text{ mg/m}^2$, which was equivalent to one tenth of the dose that would kill 10% of mice (LD10). This dose was then increased by 100%, unless grade 2 or higher toxicity was observed according to the European Organization for Research and Treatment of Cancer criteria." Webb

defined the maximum tolerated dose as that which caused grade 3 or 4 toxicity in at least 50% of patients.

Samples of blood, bone marrow, and fine-needle aspirates of lymph nodes were collected at weeks 0 (start of treatment), 2, and 6. Mononuclear cells, freshly separated by Ficoll-Isopaque centrifugation, were suspended in 10% dimethyl sulfoxide and stored in liquid nitrogen. At the time of analysis, the samples were fixed in 70% ethanol, incubated with an antibody to the BCL-2 protein (DAKO, clone 124) followed by further incubation with anti-immunoglobulin G labelled with fluorescein isothiocyanate. Levels of BCL-2 protein were measured by flow cytometry of gated lymphocytes. Within this population of cells, those positive and negative for BCL-2 were identified. The mean (SD) levels of BCL-2 protein were calculated by gating on those positive for BCL-2. All samples from each patient were labelled simultaneously under the same conditions. Because changes in the concentration of BCL-2 could reflect general changes in all protein expression, we used non-specific changes in another protein (from patient 3 onwards) as the control; these levels were measured by flow cytometry and samples were incubated with fluorescein-conjugated HLA-A, B, C antibody. The levels of HLA were consistent between individual samples for each patient.

Toxic effects of treatment

Dose escalation at 100% increments was possible, as planned, due to low toxicity. There was no antisense-related haematological toxicity (table 2, *not included in this quotation*). However, patient 8 developed grade 3 leucopenia and grade 2 thrombocytopenia associated with a Haemophilus influenzae chest infection at the start of treatment.

Antisense treatment was continued in patient 8, and leucopenia and thrombocytopenia resolved after treatment with intravenous antibiotics, which suggested a non-oligonucleotide effect. Patient 9 developed grade 2 thrombocytopenia at the end of the 2nd week, and moderate (up to 31%)

eosinophilia was observed. Subsequently, at week 6, infiltration of bone marrow and progressive disease in lymph nodes was observed. Because the thrombocytopenia and eosinophilia resolved with subsequent chemotherapy, these effects were more likely to result from advanced-stage lymphoma than from the antisense oligonucleotide. Lymphopenia was present in four patients (patients 3, 7, 8, and 9) at the start of treatment and did not worsen during antisense therapy. Anaemia was observed in three patients (patients 2, 5, and 9), but was not dose related and seemed to be associated with advanced infiltration of bone marrow. No clotting abnormalities (prothrombin or partial thromboplastin times or fibrinogen) or treatment-related changes in the CD4/CD8 ratio were observed. Repeated samples of bone marrow aspirates and urines showed no evidence of treatment-related aplasia.

Non-haematological toxic effects are shown in table 2 (*not included in this quotation*). Patient 7 had episodes of transient syncope during rest 2 days after the end of treatment. These episodes were caused by obstruction of the superior vena cava due to progressive mediastinal disease. After chemotherapy to reduce this obstruction, no further episodes have occurred. All nine patients had a transient increase in non-fasting blood glucose concentrations of glucose, but none exceeded 12 mmol/L and all patients' blood glucose concentrations returned to within the normal range after stopping antisense therapy. No intervention was required and the degree of hyperglycaemia was not dose related. Four patients developed an infection but no infection was directly attributable to the antisense therapy. Antisense treatment had no effect on liver function. The only significant toxic effect of antisense therapy was a local skin reaction around the infusion site. In eight patients this reaction simply required retiling of the line every 3-4 days. However, one patient (patient 4) had a local inflammatory reaction that became unacceptably painful about 12 h after the start of treatment. A skin biopsy sample from the inflamed area showed perivascular and periductal infiltrate in the dermis, consisting of T lymphocytes (unable to identify subtype), histiocytes, and a few plasma cells. The epidermis was normal and there was no vasculitis. Despite several site changes and reduction of drug concentration by 50%, the

inflammation persisted and treatment was stopped. Two patients who received the same dose and three who received a 100% dose increment did not have the same degree of reaction as patient 4.

The only significant toxic effect of BCL-2 antisense therapy was an inflammatory response at the injection site. In one patient this response was severe enough for the treatment to be stopped. However, response of this severity did not occur in the other patients, even at higher doses. We also noted transient non-fasting hyperglycaemia during the treatment period. The effect had no clinical implications, but has already been reported by Bishop and colleagues...which suggests that it may be associated with the phosphorothioate backbone chemistry and not with the dose. These investigators also reported a transient increase in concentrations of liver aminotransferases in two patients, which resolved after the completion of treatment...Another trial (targeting HIV) of phosphorothioates given over 2 h noted a transient increase in partial thromboplastin times not associated with clotting deficiency...By contrast, we did not find any changes in clotting factors or in concentrations of liver aminotransferases."

The full text of the above-quoted report is available from the publisher of The Lancet or through its web site at www.thelancet.com.

EXAMPLE VI

SUPPRESSION OF IgE PRODUCTION IN ANIMALS IMMUNIZED ACCORDING TO THE INVENTION

Serum antigen-specific IgB levels were measured by in the animals immunized as described in Example 1. As shown in Figure 5, high titers of IgE (5000 U/ml) were produced in the mice immunized with antigen and CT, while no detectable titers of IgE were produced in the mice immunized with ISS-ODN and antigen, whether by intranasal or intradermal routes.

EXAMPLE VII SYSTEMIC IL-4, IL-5, IL-10 AND INF- γ LEVELS, AND CD4+ LYMPHOCYTE PROLIFERATION, IN MICE AFTER DELIVERY OF ISS-ODN WITHOUT ANTIGEN

To determine the effect of ISS-ODN independent of antigen, BALB/c mice were injected intravenously with 100 μ g of DY1018, DY1019 or a random sequence control (DY1043) then sacrificed 24 hrs later. Splenocytes were harvested from each mouse.

96 well microtiter plates were coated with anti-CD3 antibody (Pharmingen, La Jolla, CA) at a concentration of 1 μ g/ml of saline. The anti-CD3 antibody stimulates T cells by delivering a chemical signal which mimics the effects of binding to the T cell receptor (TCR) complex. The plates were washed and splenocytes added to each well (4x10⁵/well) in a medium of RPMI 1640 with 10% fetal calf serum. Supernatants were obtained at days 1, 2 and 3.

Th2 cytokine (IL-4, IL-5 and IL-10) levels were assayed in the supernatants using a commercial kit; Th1 cytokine (INF- γ) levels were assayed with an anti-INF- γ murine antibody assay (see, e.g., Coligan, "Current Protocols in Immunology", Unit 6.9.5., Vol. 1, Wiley & Sons, 1994). Relatively high levels of IL-4 and IL-10 with low levels of INF- γ would be expected in mice with a Th2 phenotype, while relatively low levels of IL-4 and IL-10 with high levels of INF- γ would be expected in mice with a Th1 phenotype. Relatively high levels of IL-5 characterize a pro-inflammatory milieu, while the converse is true of relatively low levels of IL-5.

As shown in Figures 6 and 7, levels of anti-CD3 stimulated IL-4 and IL-10 secretion in DY1018 treated mice were substantially lower than in the control mice. Levels in the DY1019 mice were intermediate. Levels of pro-inflammatory IL-5 were reduced in DY1018 treated mice to a comparable extent (Figure 8).

- Levels of T cell proliferation in response to antigen challenge were greatly reduced in DY1018 (ISS-ODN) treated mice as compared to DY1019 (mutant ISS-ODN) treated and control mice. This suppression of T cell proliferation was reversible on administration of IL-2, demonstrating that the suppression was due to Th2 anergy in the ISS-ODN treated mice (see, Table below).

Treatment	Control (CPM)	ISS-ODN (CPM)	M-ODN (CPM)
OVA (50 µg/ml)	40680 ± 5495	15901 ± 4324	42187 ± 13012
OVA + IL-2 (1.5 ng/ml)	65654 ± 17681	42687 ± 6329	79546 ± 10016
OVA + IL-2 (15 ng/ml)	60805 ± 19181	57002 ± 10658	60293 ± 5442

- Levels of Th1 stimulated IFN- γ secretion were greatly increased in the DY1018 treated mice, but substantially reduced in the DY1019 treated mice (as compared to the control), indicating stimulation of a Th2-type milieu in the latter mice (Figure 9). Additional data demonstrating these results are shown in the Table below. "b/f" in the Table refers to before, "1st" and "2nd" refer to administration of the compound before the 1st or 2nd antigen challenge.

- Importantly, treatment of mice before antigen challenge is even more effective in shifting the immune response on antigen challenge to a Th1 phenotype than is post-challenge treatment. As shown in Figures 10 and 11, antigen primed (but unchallenged) animals injected with ISS-ODN DY1019 72 hours before antigen challenge (with β galactosidase) mounted a more robust Th1-type immune response to the antigen than did their post-challenge treated littermates or littermates treated pre-challenge with a mutant, inactive oligonucleotide (DY1019), as measured by increased IFN γ secretion (Figure 10) and CD4+ lymphocyte proliferation (Figure 11).

Set #	IL-6 (pg/ml)	IFN- γ (pg/ml)

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Set #	IL-6 (pg/ml)	IFN- γ (pg/ml)
1 (naive)	4.20	4.20
2 (ISS) in b/f 1st	466 ± 40	246 ± 86
3 (ISS) in b/f 2nd	531 ± 109	168 ± 22
4 (ISS) in with 2nd	575 ± 90	98 ± 44
5 (ISS) in b/f each	200 ± 66	443 ± 128
6 (ISS) ip: b/f 1st	190 ± 52	664 ± 61
7 (ISS) ip: b/f 2nd	421 ± 102	252 ± 24
8 (ISS) ip with 2nd	629 ± 110	104 ± 15
9 (ISS) ip: b/f each	121 ± 18	730 ± 99
10 (ISS) it: b/f each	191 ± 49	610 ± 108
11 (M-ISS) in: b/f each	795 ± 138	31 ± 22
12 (M-ISS) ip: b/f each	820 ± 122	33 ± 33
13 (M-ISS) it: b/f each	657 ± 52	102 ± 57
14 (tetroid) ec: b/f each	424 ± 90	4.20
15 (tetroid) ec: daily	252 ± 96	4.20

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Set #	IL-5 (pg/ml)	IFN- γ (pg/ml)
16 (control)	750 \pm 124	24 \pm 21
not treated		

Further, ISS-ODN administered according to the invention suppress Th2 cytokine release from Th2-sensitized mouse cells (splenocytes harvested from OVA-primed mice, then incubated for 72 hours with 100 μ g/ml OVA *in vitro*). ISS-ODN treatment took place either 1 (-1) or 3 (-3) days before sacrifice. These data are shown below:

Group	IL-3 (pg/ml)	IL-5 (pg/ml)	IFN- γ (pg/ml)
Control	1299 \pm 89	657 \pm 52	4 20
ISS-ODN (-1)	309 \pm 26	112 \pm 18	4 20
ISS-ODN (-3)	463 \pm 48	144 \pm 27	4 20
ISS-ODN (-1)	964 \pm 81	508 \pm 77	4 20

SEQUENCE LISTING

SEQUENCE ID Nos. 1 through 18 are representative hexameric nucleotide sequences of ISS-ODN.

SEQUENCE ID No. 19 is the complete nucleotide sequence of ISS-ODN DY1018.

5 SEQUENCE ID No. 20 is the complete nucleotide sequence of an inactive ISS-ODN mutant, DY1019.

CLAIMS

The invention claimed is:

1. A method for inducing mucosal immunity to an antigen in a mammalian host, including the production of secretory IgA (sIgA), comprising introducing an immunostimulatory oligonucleotide (ISS-ODN) and the antigen into host mucosa, wherein the ISS-ODN includes a core nucleotide sequence having the formula: 5'-Purine-Purine-[C][G]-Pyrimidine-Pyrimidine-3'.

2. The method according to Claim 1 wherein the level of sIgA production induced in the host is enhanced as compared to the magnitude of sIgA production achievable in response to the antigen alone.

3. The method according to Claim 2 wherein the enhanced sIgA production in the host includes antigen-specific sIgA.

4. The method according to Claim 1 wherein the sIgA production occurs in the mucosal tissue into which the ISS-ODN was introduced.

5. The method according to Claim 4 wherein the sIgA production occurs in mucosal tissue distant from the tissue into which the ISS-ODN was introduced.

6. The method according to Claim 1 wherein the core nucleotide sequence is selected from the group of sequences consisting of any of SEQ ID Nos. 2-18.

7. The method according to Claim 1 wherein the core nucleotide sequence consists of SEQ ID No. 1.

8. The method according to Claim 7 wherein the ISS-ODN nucleotide sequence consists of SEQ ID No. 19.

9. The method according to Claim 1 wherein the ISS-ODN and antigen are introduced into the host after the host has been separately exposed to the antigen.

10. The method according to Claim 1 wherein the ISS-ODN and antigen are introduced into the host before the host has been separately exposed to the antigen.

11. The method according to Claim 1 wherein the antigen is introduced into the same host mucosal tissue into which the ISS-ODN is introduced.

12. The method according to Claim 1 wherein the ISS-ODN is conjugated to the antigen.

13. The method according to Claim 1 wherein the induction of mucosal immunity in the host is accompanied by host production of cytotoxic T lymphocytes.

14. The method according to Claim 1 wherein the induction of mucosal immunity in the host is accompanied by biasing of the host immune response toward a Th1 phenotype, wherein development of the Th1 phenotype is evidenced by any of the following host immune responses:

(i) a reduction in levels of IL-4 measured before and after antigen-challenge; or detection of lower levels of IL-4 in a treated host as compared to an antigen-primed, or primed and challenged, control;

(ii) an increase in levels of IL-12, IL-18 and/or IFN (α , β or γ) before and after antigen challenge; or detection of higher levels of IL-12, IL-18 and/or IFN (α , β or γ) in an ISS-ODN treated host as compared to an antigen-primed or, primed and challenged, control;

(iii) IgG2a antibody production in a treated host; or

- (iv) a reduction in levels of antigen-specific IgE as measured before and after antigen challenge; or detection of lower levels of antigen-specific IgE in an ISS-ODN treated host as compared to an antigen-primed, or primed and challenged, control.

15. The method according to Claim 1 wherein the host mucosa into which the ISS-ODN is introduced is respiratory tissue.

16. The method according to Claim 15 wherein the ISS-ODN is introduced into the respiratory tissue through the host nasal passages.

17. A method for inducing mucosal immunity to an antigen in a mammalian host, including the production of secretory IgA, comprising introducing an immunostimulatory oligonucleotide (ISS-ODN) into host mucosa in the presence of the antigen, wherein the ISS-ODN includes a core nucleotide sequence having the formula: 5'-Purine-Purine-[C]-[O]-Pyrimidine-Pyrimidine-3', wherein the level of sIgA production induced in the host is at least three times the magnitude of sIgA production achievable in response to introduction of the antigen alone into the mucosal tissue.

18. The method according to Claim 17 wherein the enhanced sIgA production in the host consists of the production of antigen-specific sIgA.

19. The method according to Claim 17 wherein the enhanced sIgA production occurs in the mucosal tissue into which the ISS-ODN was introduced.

20. The method according to Claim 19 wherein the enhanced sIgA production occurs in mucosal tissue distant to the mucosal tissue into which the ISS-ODN was introduced.

21. The method according to Claim 17 wherein the core nucleotide sequence is selected from the group of sequences consisting of any of SEQ.ID Nos. 2-18.

22. The method according to Claim 18 wherein the core nucleotide sequence consists of SEQ.ID.No.1.

23. The method according to Claim 22 wherein the ISS-ODN nucleotide sequence consists of SEQ.ID.No. 19.

24. The method according to Claim 17 wherein the ISS-ODN and antigen are introduced into the host after the host has been separately exposed to the antigen.

25. The method according to Claim 17 wherein the ISS-ODN and antigen are introduced into the host before the host has been separately exposed to the antigen.

26. The method according to Claim 17 wherein the antigen is introduced into the same host mucosal tissue into which the ISS-ODN is introduced.

27. The method according to Claim 17 wherein the ISS-ODN is conjugated to the antigen.

28. The method according to Claim 17 wherein the induction of mucosal immunity in the host includes host production of cytotoxic T lymphocytes.

29. The method according to Claim 17 wherein the induction of mucosal immunity in the host is accompanied by biasing of the host immune response toward a Th1 phenotype, wherein development of the Th1 phenotype is evidenced by any of the following host immune responses:

(i) a reduction in levels of IL-4 measured before and after antigen-challenge; or detection of lower levels of IL-4 in a treated host as compared to an antigen-primed, or primed and challenged, control;

(ii) an increase in levels of IL-12, IL-18 and/or IFN (α , β or γ) before and after antigen challenge; or detection of higher levels of IL-12, IL-18 and/or IFN

(α , β or γ) in an ISS-ODN treated host as compared to an antigen-primed or, primed and challenged, control;

(iii) IgG2a antibody production in a treated host; or

(iv) a reduction in levels of antigen-specific IgE as measured before and after antigen challenge; or detection of lower levels of antigen-specific IgE in an ISS-ODN treated host as compared to an antigen-primed, or primed and challenged, control.

30. The method according to Claim 17 wherein the host mucosa into which the ISS-ODN is introduced is respiratory tissue.

31. The method according to Claim 29 wherein the ISS-ODN is introduced into the respiratory tissue through the host nasal passages.

32. A method for inducing mucosal immunity to an antigen in a mammalian host, including the production of secretory IgA, comprising introducing an immunostimulatory oligonucleotide (ISS-ODN) into host mucosa in the presence of the antigen, wherein the ISS-ODN includes a core nucleotide sequence having the formula: 5'-Purine-Purine-[C]-[G]-Pyrimidine-Pyrimidine-3', wherein the level of sIgA production induced in the host is equivalent or greater than the magnitude of sIgA production achievable in response to introduction of the antigen and cholera toxin adjuvant into the mucosal tissue.

33. The method according to Claim 32 wherein the enhanced sIgA production in the host consists of the production of antigen-specific sIgA.

34. The method according to Claim 32 wherein the enhanced sIgA production occurs in the mucosal tissue into which the ISS-ODN was introduced.

35. The method according to Claim 34 wherein the enhanced sIgA production occurs in a mucosal tissue distant to the mucosal tissue into which the ISS-ODN was introduced.

36. The method according to Claim 32 wherein the core nucleotide sequence is selected from the group of sequences consisting of any of SEQ ID Nos. 2-18.

37. The method according to Claim 32 wherein the core nucleotide sequence consists of SEQ ID No. 1.

38. The method according to Claim 37 wherein the ISS-ODN nucleotide sequence consists of SEQ ID No. 19.

39. The method according to Claim 32 wherein the ISS-ODN and antigen are introduced into the host after the host has been separately exposed to the antigen.

40. The method according to Claim 32 wherein the ISS-ODN and antigen are introduced into the host before the host has been separately exposed to the antigen.

41. The method according to Claim 32 wherein the antigen is introduced into the same host mucosal tissue into which the ISS-ODN is introduced.

42. The method according to Claim 32 wherein the ISS-ODN is conjugated to the antigen.

43. The method according to Claim 32 wherein the induction of mucosal immunity in the host includes host production of cytotoxic T lymphocytes.

44. The method according to Claim 32 wherein the induction of mucosal immunity in the host is accompanied by biasing of the host immune response toward a Th1 phenotype, wherein development of the Th1 phenotype is evidenced by any of the following host immune responses:

(i) a reduction in levels of IL-4 measured before and after antigen-challenge; or detection of lower levels of IL-4 in a treated host as compared to an antigen-primed, or primed and challenged, control;

(ii) an increase in levels of IL-12, IL-18 and/or IFN (α , β or γ) before and after antigen challenge; or detection of higher levels of IL-12, IL-18 and/or IFN (α , β or γ) in an ISS-ODN treated host as compared to an antigen-primed or, primed and challenged, control;

(iii) IgG2a antibody production in a treated host; or

(iv) a reduction in levels of antigen-specific IgE as measured before and after antigen challenge; or detection of lower levels of antigen-specific IgE in an ISS-ODN treated host as compared to an antigen-primed, or primed and challenged, control.

45. The method according to Claim 32 wherein the host mucosa into which the ISS-ODN is introduced is respiratory tissue.

46. The method according to Claim 45 wherein the ISS-ODN is introduced into the respiratory tissue through the host nasal passages.

47. A pharmaceutical composition comprising (a) an immunostimulatory oligonucleotide (ISS-ODN) conjugated to an antigen, wherein the ISS-ODN includes a core nucleotide sequence having the formula: 5'-Purine-Purine-[C]-[G]-Pyrimidine-3'; and, (b) a promoter of drug absorption into mucosal tissue.

48. A pharmaceutical composition comprising (a) an immunostimulatory oligonucleotide (ISS-ODN), wherein the core nucleotide sequence of the ISS-ODN is selected from the group of core sequences of SEQ.ID.Nos. 1 through 18; and, (b) a promoter of drug absorption into mucosal tissue.

49. The pharmaceutical composition of Claim 48, wherein the ISS-ODN consists of SEQ.ID.No.19.

50. The pharmaceutical composition of Claim 48, further comprising an antigen.

51. A kit for use in inducing mucosal immunity in a mammalian host comprising an immunostimulatory oligonucleotide (ISS-ODN) conjugated to an antigen within a sterile vial and a device for delivering the ISS-ODN into host mucosal tissue.

52. A kit for use in inducing mucosal immunity in a mammalian host comprising an immunostimulatory oligonucleotide (ISS-ODN) within a sterile vial and a device for delivering the ISS-ODN into host mucosal tissue.

53. The kit of Claim 52, wherein the ISS-ODN consists of SEQ.ID.No.19.

54. The kit of Claim 52, further comprising an antigen.

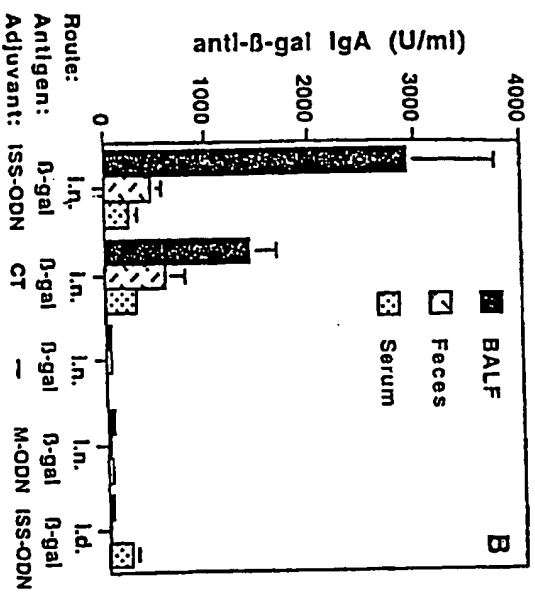


Fig. 1a

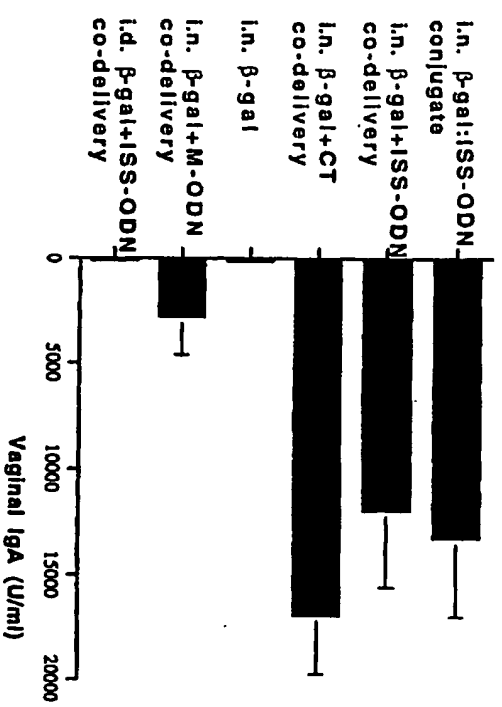


Fig. 1b

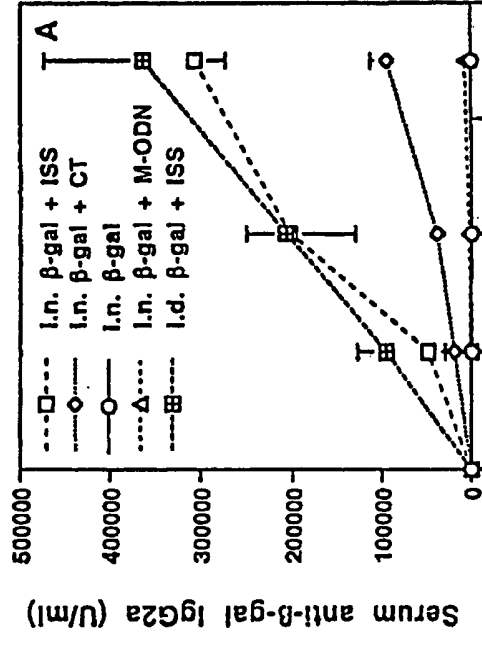


Fig. 3a

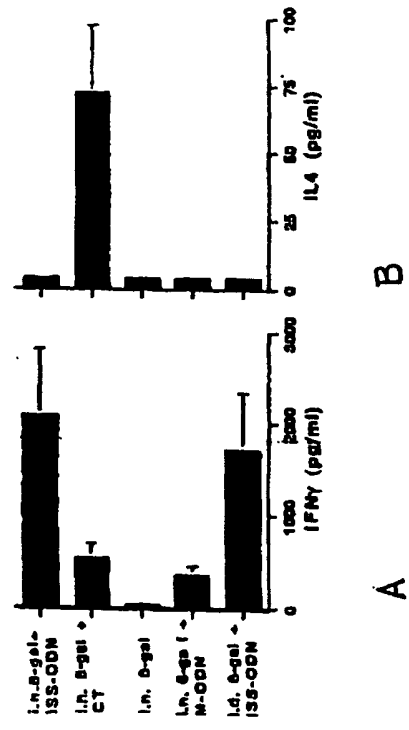


Fig. 2

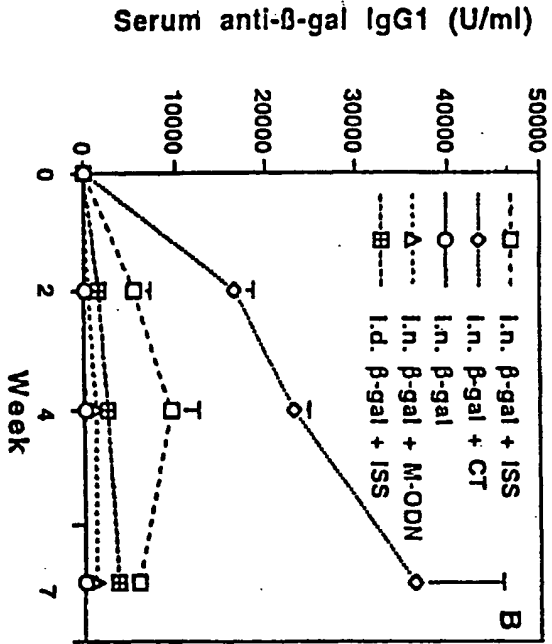


Fig. 3b

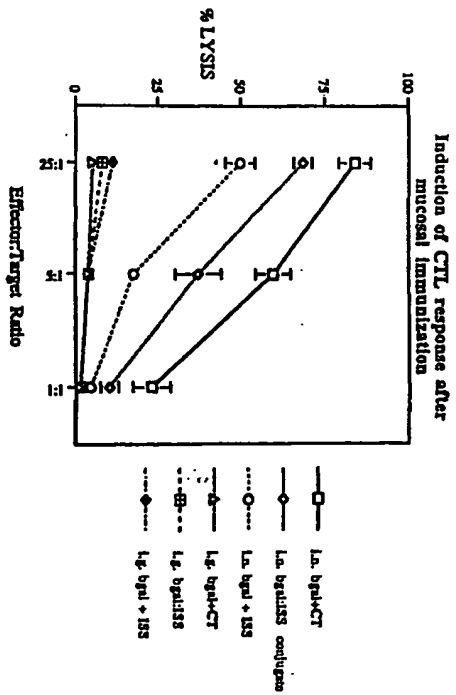


Fig. 4

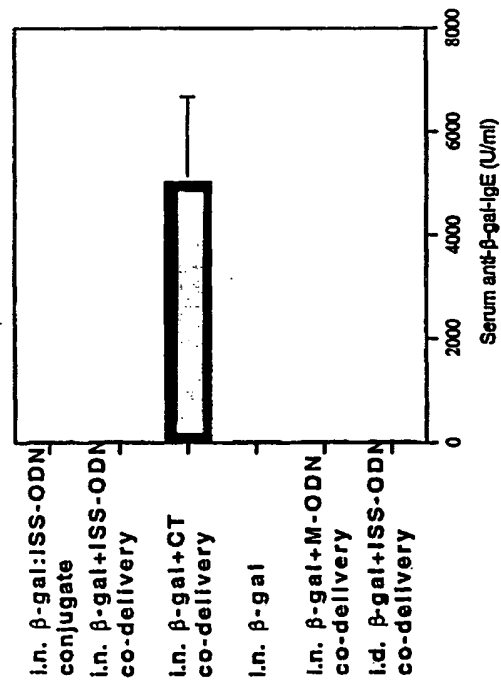


Fig. 5

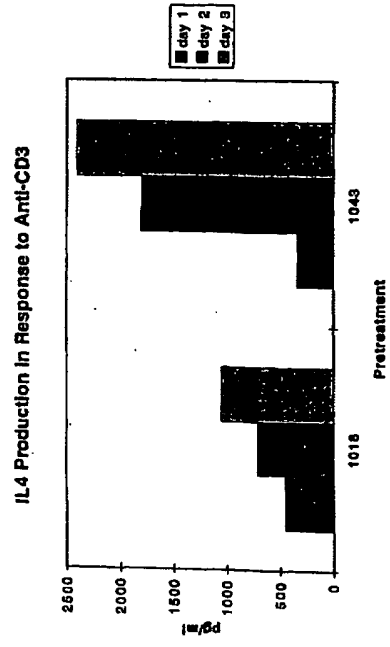


FIGURE 6

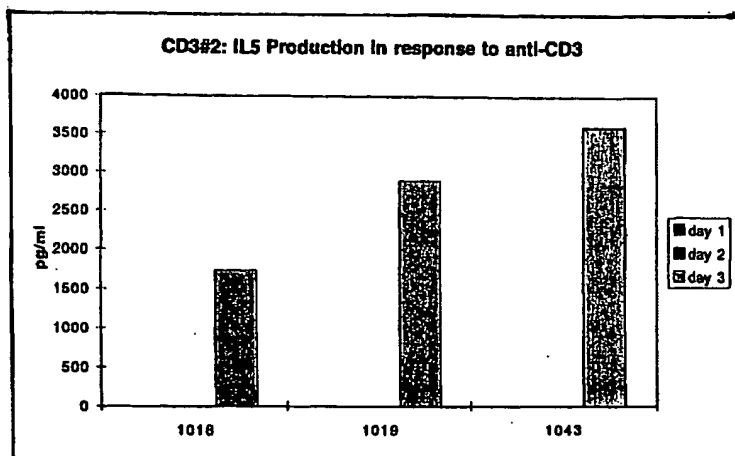


FIGURE 7

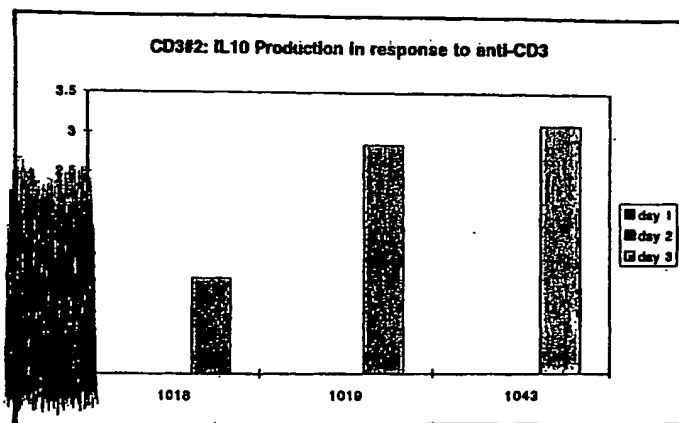


FIGURE 8

IFN γ Production in Response to Anti-CD3

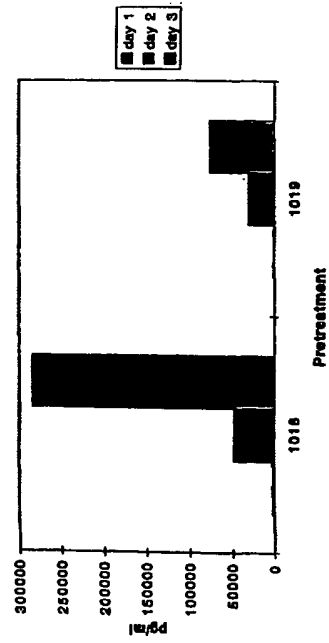
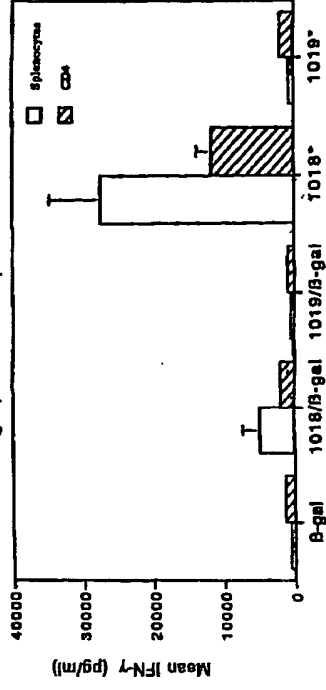


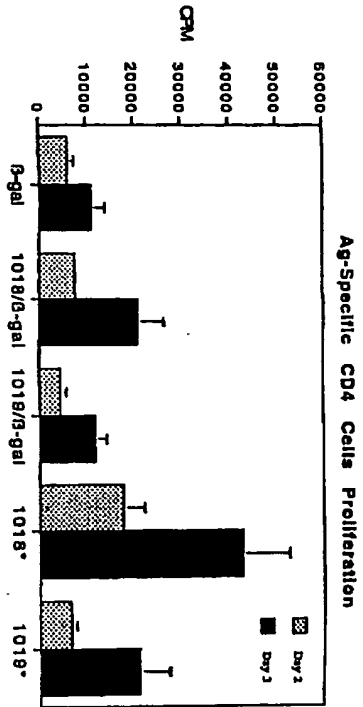
FIGURE 9

Ag-Specific IFN- γ Production



*Oligonucleotides were injected 72 hours prior to B-gal at the same site.

FIGURE 10



Oligonucleotides were injected 72 hours prior to B-gal injection at the same site.

FIGURE 14

SEQUENCE LISTING

(1) GENERAL INFORMATION

(1) APPLICANT: The Regents of the University of California

(1) TITLE OF THE INVENTION: METHODS AND ADVANTAGE FOR STIMULATING IMMUNITY

(11) NUMBER OF SEQUENCES: 20

(14) CORRESPONDENCE ADDRESS:

(A) ADDRESSER: Pulbright & Jaworski, L.L.P.

(B) STREET: 865 S. Figueroa Street, 19th Floor

(C) CITY: Los Angeles

(D) STATE: CA

(E) COUNTRY: US

(F) ZIP: 90017

(V) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette

(B) COMPUTER: IBM Compatible

(C) OPERATING SYSTEM: Windows 95

(D) SOFTWARE: FASTSEQ for Windows Version 2.0

(V4) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: 09/____

(B) FILING DATE: _____

(C) CLASSIFICATION: _____

(V41) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: 08/927,130

(B) FILING DATE: 05 September 1997

(V411) ATTORNEY/AGENT INFORMATION:

(A) NAME: Berlinet, Robert

(B) REGISTRATION NUMBER: 20,121

(C) REFERENCE/DOCKET NUMBER: 5555-188

(V42) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 213-892-9200

(B) TELEFAX: 213-860-4518

(2) INFORMATION FOR SEQ ID NO.1:

(1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: linear

(11) MOLECULE TYPE: non-coding oligonucleotides

(X1) SEQUENCE DESCRIPTION: SEQ ID NO.1:

6 AACGTT

(2) INFORMATION FOR SEQ ID NO.2:

(1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: linear

(11) MOLECULE TYPE: non-coding oligonucleotides

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:2:

5 AGCGTC

(2) INFORMATION FOR SEQ ID NO:3:

(1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: linear

(11) MOLECULE TYPE: non-coding oligonucleotides

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:3:

10 GACGTC

(2) INFORMATION FOR SEQ ID NO:4:

(1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: linear

(11) MOLECULE TYPE: non-coding oligonucleotides

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:4:

20 GCGCTC

(2) INFORMATION FOR SEQ ID NO:5:

(1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: linear

(11) MOLECULE TYPE: non-coding oligonucleotides

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:5:

30 AACGTC

(2) INFORMATION FOR SEQ ID NO:6:

(1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: linear

(11) MOLECULE TYPE: non-coding oligonucleotides

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:6:

40 AGCGTC

AGCGTC

(2) INFORMATION FOR SEQ ID NO:7:

(1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: linear

(11) MOLECULE TYPE: non-coding oligonucleotides

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:7:

10 GACGTC

(2) INFORMATION FOR SEQ ID NO:8:

(1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: linear

(11) MOLECULE TYPE: non-coding oligonucleotides

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:8:

20 GCGCTC

(2) INFORMATION FOR SEQ ID NO:9:

(1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: linear

(11) MOLECULE TYPE: non-coding oligonucleotides

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:9:

30 AACGTC

(2) INFORMATION FOR SEQ ID NO:10:

(1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: linear

(11) MOLECULE TYPE: non-coding oligonucleotides

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:10:

40 AGCGTC

(2) INFORMATION FOR SEQ ID NO:11:

(1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(11) MOLECULE TYPE: non-coding oligonucleotides

(*1) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GACGCC

6

(2) INFORMATION FOR SEQ ID NO:12:

(1) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(11) MOLECULE TYPE: non-coding oligonucleotides

(*1) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GGCGCC

6

(2) INFORMATION FOR SEQ ID NO:13:

(1) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(11) MOLECULE TYPE: non-coding oligonucleotides

(*1) SEQUENCE DESCRIPTION: SEQ ID NO:13:

AGCGCT

6

(2) INFORMATION FOR SEQ ID NO:14:

(1) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(11) MOLECULE TYPE: non-coding oligonucleotides

(*1) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GACGCT

6

(2) INFORMATION FOR SEQ ID NO:15:

(1) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(11) MOLECULE TYPE: non-coding oligonucleotides

(*1) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GACGCT

6

GCGCGT

6

(2) INFORMATION FOR SEQ ID NO:16:

(1) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(11) MOLECULE TYPE: non-coding oligonucleotides

(*1) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TTCCGA

6

(2) INFORMATION FOR SEQ ID NO:17:

(1) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(11) MOLECULE TYPE: non-coding oligonucleotides

(*1) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GCGCGT

6

(2) INFORMATION FOR SEQ ID NO:18:

(1) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(11) MOLECULE TYPE: non-coding oligonucleotides

(*1) SEQUENCE DESCRIPTION: SEQ ID NO:18:

AACGCC

6

(2) INFORMATION FOR SEQ ID NO:19:

(1) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(11) MOLECULE TYPE: non-coding oligonucleotides

(*1) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TGAAGTGTGAA CATTGCAAGAT GA

22

(2) INFORMATION FOR SEQ ID NO:20:

(1) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs

(11) MOLECULE TYPE: non-coding oligonucleotides

(*1) SEQUENCE DESCRIPTION: SEQ ID NO:20:

(A) LENGTH: 22 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(11) MOLECULE TYPE: non-coding oligonucleotides

(12) SEQUENCE DESCRIPTION: SEQ ID NO:20:

TCACCTGTGAA GCTTGAGAT GA

22

INTERNATIONAL SEARCH REPORT

International application No. PCT/US99/21203	
A. CLASSIFICATION OF SUBJECT MATTER IPC7: A61K 4800; C12N 1300 US CL: 51444; 435455 According to International Patent Classification (IPC) or to both national classification and IPC	
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) U.S.: 51444; 435455	
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched NONE	
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) AFS, nucleic acid databases	
C. DOCUMENTS CONSIDERED TO BE RELEVANT	
Category*	Citation of document, with indication, where appropriate, of the relevant passages Relevant to claim No.
X	WO 98/16247 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 23 April 1998, see entire document, especially pages 15-17, 27, 36, and 40-54.
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Date of the actual completion of the international search 23 DECEMBER 1999	
Date of mailing of the international search report 24 JAN 2000	
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